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An investigation of the inter-clonal variation of the interactive effects of cadmium and *Microcystis aeruginosa* on the reproductive performance of *Daphnia magna*

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38 Abstract

39 Interactive effects between chemical and natural stressors as well as genetically determined variation
40 in stress tolerance among individuals may complicate risk assessment and management of chemical
41 pollutants in natural ecosystems. Although genetic variation in tolerance to single stressors has been
42 described extensively, genetic variation in interactive effects between two stressors have only rarely
43 been investigated. Here, we examined the interactive effects between a chemical stressor (Cd) and a
44 natural stressor (the cyanobacteria *Microcystis aeruginosa*) on the reproduction of *Daphnia magna* in
45 20 genetically different clones using a full-factorial experimental design and with the independent
46 action model of joint stressor action as the reference theoretical framework. Across all clones, the
47 reduction of 21-day reproduction compared to the control treatment (no Cd, no *M. aeruginosa*) ranged
48 from -10% to 98% following Cd exposure alone, from 44% to 89% for *Microcystis* exposure alone, and
49 from 61% to 98% after exposure to Cd + *Microcystis* combined. Three-way ANOVA on log-
50 transformed reproduction data of all clones together did not detect a statistically significant Cd ×
51 *Microcystis* interaction term (F-test, $p=0.11$), meaning that *on average* both stressors do not interact in
52 inhibiting reproductive performance of *D. magna*. This finding contrasted expectations based on some
53 known shared mechanisms of toxicity of Cd and *Microcystis* and therefore cautions against making
54 predictions of interactive chemical + natural stressor effects from incomplete knowledge on affected
55 biological processes and pathways. Further, still based on three-way ANOVA, we did not find
56 statistically significant clone × Cd × *Microcystis* interaction when data for all clones were analyzed
57 together (F-test, $p=0.07$), suggesting no inter-clonal variation of the interactive effect between Cd and
58 *Microcystis*. However, when the same data were quantitatively analyzed on a clone-by-clone scale, we
59 found a relatively wide range of deviations between observed and IA-model-predicted reproduction in
60 combined Cd + *Microcystis* treatments (both in direction and magnitude), suggesting some biological
61 significance of inter-clonal variation of interactive effects. In one of the twenty clones this deviation was
62 statistically significant (two-way ANOVA, F-test, $p=0.005$), indicating an interactive Cd × *Microcystis*
63 effect in this clone. Together, these two observations caution against the extrapolation of conclusions
64 about mixed stressor data obtained with single clones to the level of the entire species and to the level
65 of natural, genetically diverse populations.

66 **Keywords:** mixture, interactive effects, cadmium, cyanobacteria, *Daphnia*, *Microcystis*

1. Introduction

The existence of interactive effects considerably complicates ecological risk assessment of chemical mixtures (Van Gestel et al., 2010). This situation may be complicated even more by the presence of natural stressors as recently stressed in a review by Holmstrup et al. (2010). They reported synergistic interactions between chemicals and natural stressors in more than 50% of the 150 ecotoxicological studies they evaluated. While several of these studies have demonstrated interactive effects on zooplankton between chemicals and important natural stressors like heat, oxygen limitation and pathogen stress, interactions with cyanobacterial stressors have received very little attention so far (Cerbin et al., 2010; Bernatowicz and Pijanowska, 2011). We argue that it is important to study such potential interactions because harmful cyanobacterial blooms have already been shown to cause major ecological problems of their own (Dao et al., 2010; Lüring et al., 2003; Falconer, 2001) and are predicted to occur with increasing frequency and severity due to climate change (Kosten et al., 2012; Paerl and Huisman, 2008; Paul, 2008; O'Neil et al., 2011).

In this context, Cerbin et al. (2010) recently reported synergistic interactive effects on a wide range of life-history characteristics in *Daphnia pulicaria* following sequential exposure to a pulse of the insecticide carbaryl followed by exposure to *Microcystis aeruginosa*. Bernatowicz and Pijanowska (2011) reported that the effect of the filamentous cyanobacteria *Cylindrospermopsis raciborskii* on *Daphnia* reproduction (but not on growth or vertical migration behavior) was modified by the presence of PCB52 (but not by PCB153). These authors also reported that the interactive effect with PCB52 differed among different clones within the *Daphnia longispina* complex, as demonstrated by a significant clone × cyanobacteria × PCB interaction. Collectively, these two studies already indicate that interactive effects between chemicals and cyanobacteria in *Daphnia* spp. are likely dependent on such factors as the nature of the chemical, the cyanobacterial species, and the genotype of the *Daphnia* clone investigated. This complexity highlights the need to continue investigation of these factors.

In this study we investigated the occurrence of interactive effects of the cyanobacterium *M. aeruginosa* combined with cadmium stress, on the reproduction of 20 different clones of the European waterflea, *Daphnia magna*. *D. magna* is a keystone crustacean in freshwater lakes and ponds; it occupies a central position in aquatic food webs as a principal grazer of algae and a primary forage for fish

(Lampert, 2011). It is frequently used as a model species in ecotoxicological studies (Altschuler et al., 2011). Because, (i) it is well-known that it is very difficult to extrapolate results of single-stressor studies performed with a single clone (or even a few clones) to genetically diverse populations (Barata et al., 1998; Barata et al., 2002a), (ii) because the same may equally well apply to results of multiple-stressor studies (Barata et al., 2002b, Bernatowicz and Pijanowska, 2011, De Coninck et al., 2013) and (iii) because research on the genetic (inter-clonal) variation of interactive effects of chemicals and natural stressors is highly underrepresented in the literature, we chose to perform our study with several genetically different clones of *D. magna*.

We chose to investigate cadmium and *M. aeruginosa* as the stressors in the present study for two reasons. First, both stressors individually, but also in combination, are ecologically and environmentally relevant. Cadmium is an ubiquitous environmental stressor that is considered to pose risks to aquatic ecosystems at regional and local scales (EU, 2007). *Microcystis* sp. is one of the most common harmful cyanobacterial bloom formers in freshwater systems all over the world, except Antarctica (Fristachi and Sinclair, 2008). The adverse effects of *Microcystis* sp. on *Daphnia* spp. are well studied and have been related to its lack of essential fatty acids or lipids (Haney et al., 1995; Lüring, 2003), presence of feeding deterrents (Demott et al., 1991; Lüring, 2003), and/or production of toxins such as microcystins and aeruginosins (Demott et al., 1991; Lüring, 2003; Rohrlack et al., 1991; O'Neil et al., 2011; van Apeldoorn et al., 2007). *Microcystis* sp. has also frequently been observed in harmful algal blooms in cadmium-contaminated lakes all over the world: from the dozens of metal-contaminated lakes in the Sudbury basin (Ontario, Canada) (Nriagu et al., 1998; Winter et al., 2011) to several lakes and lagoons in developing countries (e.g., the Lagos lagoon in Nigeria; Adesalu and Nwankwo, 2010; Ehi-Eromosele and Okiei, 2012).

Second, based on what is currently known about their mechanisms of toxicity, we expected that interactions could occur between cadmium and *Microcystis* stress. Both *Microcystis* (Lüring, 2003; van Apeldoorn et al., 2007; Asselman et al., 2012) and Cd (McGeer et al., 2012) are known to affect a very diverse set biological pathways or processes in aquatic organisms, and while some of these are relatively specific to each stressor (e.g., inhibition of protein-phosphatases by *Microcystis* and Ca antagonism by Cd), other pathways or processes are well-known to be shared among both stressors. For instance, both cadmium and *Microcystis* are well-known to induce oxidative stress and both have been suggested to do so by influencing the Nrf2-pathway (Shanker, 2008; Amado and Monserrat,

2010; Jos et al., 2005; Pflugmacher, 2004; Chen and Shaikh, 2009; He et al., 2008; Stewart et al., 2003). In addition, both *Microcystis* and cadmium exposure have been shown to affect digestive processes in *Daphnia*, possibly by affecting production or activity of important digestive enzymes such as trypsins (Asselman et al., 2012; De Coen and Janssen, 1997; Munger et al. 1999; Schwarzenberger et al., 2010). At least for the toxicity of binary mixtures of chemicals it is well-documented that many of the reported cases of interactive effects have been observed when both chemicals target similar biological pathways or processes (Deneer, 2000; Van Gestel et al., 2010). Hence, given that Cd and *Microcystis* are known to affect some similar biological pathways or processes (e.g., oxidative stress and digestive activity), interactive effects between them are not unlikely.

Based on the information presented above, we hypothesized that (1) combined exposure to cadmium and *M. aeruginosa* would affect reproduction in *D. magna* in an interactive manner, and (2) that this interactive effect would be dependent on the *D. magna* genotype investigated. These hypotheses were investigated experimentally using a full three-factorial experimental design with cadmium (absent or present), *M. aeruginosa* (absent or present) and *D. magna* genotype (N=20) as factors. This experimental design is appropriate for testing both hypotheses under the theoretical framework of the independent action (IA) model of joint stressor action (Van Gestel et al., 2010; De Coninck et al., 2013), but not under the framework of the concentration addition (CA) model, which would require full dose-response curves to be established for each individual stressor (Van Gestel et al., 2010). It has been proposed that CA and IA might be more appropriate for mixtures of stressors with common toxicity mechanisms and different toxicity mechanisms, respectively (Van Gestel et al., 2010). Given that, (i) *not all* mechanisms of toxicity are shared among Cd and *Microcystis* (see above) and that not all mechanisms are *exactly* known (despite what is known), and (ii) that it has also been argued that CA and IA are just two different mathematical descriptions of mixture toxicity that can always be tested, regardless of knowledge of shared or non-shared toxicity mechanisms (Jonker et al., 2005); the IA model is an appropriate theoretical framework to test here. The hypotheses put forward above were statistically tested with two-way and three-way ANOVA on log-transformed reproduction data. This is an appropriate technique to detect *statistically* significant deviations from the reference model of independent action (IA) of combined stressors, and the criterion to decide about “interactive effects” in this approach (and in our study) is the finding of *statistically* significant ANOVA interaction terms

(Sih et al., 1998; Fournier et al., 2006; De Coninck et al., 2013) (see *Materials and Methods* for details).

2. Materials and methods

2.1. Experimental organisms

All *D. magna* clones used in the present study originate from nine different natural populations in Flanders, Belgium (Table 1). All clones were cultured in modified M4-medium at $20 \pm 1^\circ\text{C}$ and under a 16h:8h light-dark cycle for several generations prior to the start of the experiment. The modified M4 medium differed from the original composition of the M4 medium (Elendt and Bias, 1990) by omitting the addition of the $\text{Na}_2\text{EDTA}+\text{FeSO}_4$ stock solution, by adding Aldrich Humic Acid (AHA) at a final concentration of 4 mg dissolved organic carbon per liter ($\text{DOC}\cdot\text{L}^{-1}$), and by reducing the water hardness to $250\text{ mg CaCO}_3\cdot\text{L}^{-1}$ while respecting the original Ca:Mg ratio. The pH of this modified M4-medium is 7.6. An AHA stock solution was prepared at a concentration of $2.5\text{ g}\cdot\text{L}^{-1}$ in a 0.001 M NaOH solution and filtered through Whatman GF/C glass microfibre filters (mesh size $1.2\text{ }\mu\text{m}$). The actual DOC concentration of the filtrate was determined with a TOC Analyzer (TOC5000, Shimadzu, Duisburg, Germany). The animals were cultured in separate glass jars per clone, each containing 10 animals and 500 mL medium. The animals were transferred to fresh medium twice a week. The cultures were fed daily to a final concentration of $5\text{ mg C}\cdot\text{L}^{-1}$ with an algae mix consisting of *Pseudokirchneriella subcapitata* and *Chlamydomonas reinhardtii* in a 3:1 cell number ratio.

2.2. Experimental design

Cultures of twenty *Daphnia magna* clonal lineages were established by hatching twenty different resting eggs collected from the sediment layer of the habitat of the nine natural populations. Individuals belonging to each clonal lineage were exposed to a control (no Cd and only the algae mix in the diet), $8\text{ }\mu\text{g}\cdot\text{L}^{-1}$ cadmium (Cd), *Microcystis aeruginosa* (MC) (50% in the diet on a $\text{mg C}\cdot\text{L}^{-1}$ basis), and the combination of both (Cd+MC) in a full factorial $20 \times 2 \times 2$ design. Exposures were performed according to OECD guideline 211 (OECD, 2008). For each clone and treatment 10 individual replicate animals less than 24 hours old were exposed in polyethylene cups containing 50 mL of the modified

M4-medium. Exposure media were spiked with $8 \mu\text{g}\cdot\text{L}^{-1}\text{CdCl}_2$ (nominal) the day prior to transferring *Daphnia* into the exposure cups, which were subsequently aerated and kept at 20°C . Exposures lasted for 21 days under controlled laboratory conditions ($20 \pm 1^\circ\text{C}$, 16h:8h light-dark cycle with a light intensity of $14 \mu\text{moles}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$). Media were renewed three times a week. Total reproduction was recorded in all replicates (i.e. the total number of juveniles produced per female over the entire test duration of 21 days).

Animals in the treatments were fed daily with an algae mix consisting of *P. subcapitata* and *C. reinhardtii* in a 3:1 cell number ratio. Animals were fed 0.125 mg C per animal in the first week, 0.25 mg C in the second week and 0.375 mg C in the last week of the exposures. The diet of the animals in the *Microcystis* and the Cd+*Microcystis* treatments consisted of 50% of the algae mix mentioned above and 50% of *M. aeruginosa* on a mg C·L⁻¹ basis. The diet in the two other treatments consisted of 100% algae mix.

The microcystin-LR producing *M. aeruginosa* strain PCC7806 was obtained from the Pasteur Culture Collection of Cyanobacteria (PCC; Paris, France) and cultured under sterile conditions in 6L culturing flasks containing 3L of BG11₀ medium (Rippka et al. 1979) under a constant light intensity of $4.34 \mu\text{moles}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ and at $20 \pm 1^\circ\text{C}$ as recommended by PCC (Paris, France). Cultures were gently aerated and allowed to grow during 10 days until mid-late log phase. Afterwards, the cyanobacteria cells were concentrated by centrifugation, washed three times and suspended in modified M4 medium before use. Dry-weight of the feeding stock suspension was determined by drying a known suspension volume at 60°C for 24 hours. Finally, the amount of carbon per liter was calculated from the dry-weight using a conversion factor of 0.5 g C per g dry wt (Geller, 1975).

2.3. Physico-chemical analyses

During the exposures, samples for determination of dissolved Cd and dissolved organic carbon (DOC) concentration were collected two times per week from freshly prepared and 48h-old medium (just after renewal of the media). All samples were filtered through a $0.45 \mu\text{m}$ Acrodisc filter (Sterlitech, Kent, USA). Samples for Cd analysis were also acidified with 1% (v/v) 14N HNO₃. All samples were stored in the dark at 4°C until analysis. Dissolved cadmium concentrations were measured using graphite

furnace atomic absorption spectroscopy (SpectrAA-100, Varian, Mulgrave, Australia) and DOC was measured using a TOC Analyzer (TOC5000, Shimadzu, Duisburg, Germany).

2.4. Data treatment and statistical analyses

All statistics were performed with Statistica 7.0 software (Statsoft, Tulsa, OK, USA). Reproduction data (number of juveniles per female) were \log_{10} -transformed prior to statistical analysis to ensure compliance with assumptions of normality (Shapiro-Wilkinson's W test) and homoscedasticity (Levene's test) and to allow Cd \times *Microcystis* interaction terms to be interpreted in relation to the IA theoretical framework (Sih et al., 1998; Fournier et al., 2006; De Coninck et al., 2013).

First, we performed three-way ANOVA on all data from all clones combined, followed by F-tests, to determine the significance of the main effects and all two-way and three-way interaction terms. All F-tests were performed at a significance level of 95% ($p < 0.05$). Of particular interest were the F-tests concerning the two-way interaction term Cd \times *Microcystis* and the three way-interaction term clone \times Cd \times *Microcystis*, as these two terms are the ones that are directly related to the two main hypotheses put forward in the *Introduction*. The finding of a significant Cd \times *Microcystis* term would indicate an effect of these two stressors that is *on average* "interactive" *across all clones*. The finding of a significant three-way clone \times Cd \times *Microcystis* term would indicate that the interactive effect between Cd and *Microcystis* differed *among clones* (see De Coninck et al., 2013, for a similar reasoning with *D. magna* clones exposed to combined carbaryl and parasite stress).

However, because three-way interactions can be interpreted in different ways (Kutner et al., 2005) and because the p-value of this term turned out to be close to 0.05 (see *Results section*), we also performed two-way ANOVA's for each clone separately to allow a more detailed evaluation of clonal differences in Cd \times *Microcystis* interactive effects (e.g., to evaluate if this interaction would be significant in one clone but not in another, or to evaluate if it would be synergistic in one clone and antagonistic in another). The finding of a significant Cd \times *Microcystis* term (at $p < 0.05$) in such a two-way ANOVA for a given clone would mean a statistically significant deviation from the IA reference model (Sih et al., 1998; Fournier et al., 2006; De Coninck et al., 2013), and thus an interactive effect for this clone.

Finally, to further evaluate the importance of considering (or not considering) multiple clones in the study of chemical \times natural stressor interactions, a quantitative comparison was made between observed reproduction ($Y_{Cd+MC,obs}$) in the combined Cd + *Microcystis* treatment and IA-model-predicted reproduction ($Y_{Cd+MC,pred}$), based on observed reproduction in the control ($Y_{control}$), the Cd alone (Y_{Cd}) and the *Microcystis* alone (Y_{MC}) treatments, following the equation reported in De Coninck et al. (2013), which was derived based on the original IA model formulation as in Bliss (1937) and Faust et al. (2003):

$$Y_{Cd+MC,pred} = Y_{Cd} \times Y_{MC} / Y_{control} \text{ (Eq.1)}$$

The predictions are plotted together with the observations in the Cd reaction norm plots for visual comparison (Figure 1).

3. Results

Dissolved Cd and DOC concentrations for freshly made and 48h old medium in all four treatments are summarized in Table 2. Both Cd and DOC concentrations did not differ significantly between treatments (Kruskal Wallis test, $p < 0.05$).

For each clone, reaction norms for reproduction as a function of Cd, in the absence or presence of *Microcystis*, are presented in Figure 1. Three-way ANOVA of the data indicated significant main effects of clone, Cd and *Microcystis* ($p < 0.001$, Table 3). Across all clones, reduction of reproduction (compared to the control treatment) ranged from -10% to 98% following Cd exposure alone, from 44% to 89% for *Microcystis* exposure alone (Figure 1). Significant clone \times Cd ($p < 0.001$) and clone \times *Microcystis* ($p = 0.019$) interactions were also observed (Table 3). Across all clones, reproduction was reduced by 61% to 98% in the combined Cd + *Microcystis* treatment compared to the control treatment (Figure 1), but no significant Cd \times *Microcystis* interaction was detected in the three-way ANOVA ($p = 0.105$). Finally, the three-way clone \times Cd \times *Microcystis* interaction was also not significant ($p = 0.073$).

A quantitative comparison of observed and IA-model predicted reproduction in the combined Cd + *Microcystis* treatment for each clone separately revealed a considerable variation of log₁₀-deviations among clones ($\log_{10} Y_{\text{obs,Cd+MC}} - \log_{10} Y_{\text{pre,Cd+MC}}$, values denoted as *d* in Figure 1): 13 were positive (observed reproduction > predicted reproduction, *trend* of antagonism) and 7 were negative (observed reproduction < predicted reproduction, *trend* of synergism), with a mean of 0.11 and ranging between -0.55 and 0.91 (Figure 1). However, when a two-way ANOVA was performed on all twenty clones separately, a significant ($p < 0.01$) Cd × *Microcystis* interaction was observed for only one of these 20 clones (i.e., for OM2-24) (p -values for all clones in Figure 1, detailed ANOVA results are available in supportive information, Table S1). As the observed reproduction in Cd + *Microcystis* treatment is larger than the IA-model-predicted reproduction (Figure 1), the interaction for this specific clone is considered antagonistic.

4. Discussion

In natural ecosystems, chemical stressors often occur together with (natural) biological stressors. As interactive effects between those two categories of stressors may considerably complicate risk assessment and management of chemical pollutants, the study of stressor combinations that may cause interactive effects is receiving considerable attention (Holmstrup et al., 2010). *Daphnia magna* is a potentially valuable model species to study such interactions as it is a highly standardized ecotoxicological test species for reproductive toxicity (OECD, 2008) and results of this type of research could eventually facilitate implementation of natural-chemical stressor interactions in environmental risk assessment and management practices. However, at the same time, it is well-known that the use of *Daphnia* ecotoxicity testing results for predicting responses of true natural populations to chemicals can be challenging (Barata et al. 1998; Messiaen et al. 2010). One important reason for this is that there is large - genetically determined - variation (in their response and tolerance to stressors) among *D. magna* clones. Consequently, the monoclonal laboratory populations of *D. magna* which are typically used for conventional toxicity testing may not reflect the response and tolerance of natural, genetically diverse populations to stressors. Differences in stress response and/or tolerance among *D. magna* clones have been demonstrated by various workers and for a range of chemicals (Barata et al. 1998, Barata et al. 2006, Baird et al. 1990, Baird et al. 1991, Coors et al. 2009, Soares et al. 1992)

and natural stressors (fish predation: Jansen et al. 2010; parasites: Ebert et al. 1998, Jansen et al. 2011; heat: Muyssen et al. 2010), including Cd (Messiaen et al., 2010; Muyssen et al., 2010; Barata et al., 2002a) and *M. aeruginosa* (Hietala et al. 1995, Lemaire et al., 2012). Our present study did not yield surprises in this regard, as it clearly confirmed these findings for Cd and *M. aeruginosa*, as demonstrated by the detection of statistically significant clone x Cd and clone x *Microcystis* interactions noted in the three-way ANOVA (Table 3).

In contrast to research on inter-clonal differences in tolerance to single stressors (i.e. clone x stressor interaction), much less research has been performed on inter-clonal differences of interactive effects between two stressors (i.e. clone x stressor x stressor interaction). For *Daphnia* spp. such inter-clonal differences of interactive effects have been reported for a Cd + Zn mixture (Barata et al., 2002b), a PCB52 + *C. raciborskii* combination (Bernatowicz and Pijanowska 2011), a Cd + heat stress (Muyssen et al., 2010), and a *C. raciborskii* + temperature stress combination (Bednarska et al., 2011). In our study with 20 *D. magna* clones, we did not detect (based on our 3-way ANOVA) a statistically significant clone x Cd x *M. aeruginosa* interaction (i.e., no overall inter-clonal difference of interactive effects of the Cd + *Microcystis* mixture) and we also did not observe an overall significant Cd x *M. aeruginosa* interactive effect (Table 3). This indicates that the combined effect of Cd and *M. aeruginosa* on *D. magna* is generally non-interactive when using IA as the reference model for joint stressor action. This contrasts with the general trend reported across species (aquatic and terrestrial) that combined effects of a natural stressor (heat, cold, drought, oxygen depletion, starvation or pathogens) with Cd are generally interactive (12 out of 13 cases, nine of which were synergistic interactions; as summarized in Holmstrup et al., 2010). Further, regarding chemical x cyanobacteria interactions, our data with Cd + *Microcystis* stress are in line with those observed for PCB153 + *C. raciborskii* stress (no interaction, Bernatowicz and Pijanowska 2011), but in contrast with findings for PCB52 + *C. raciborskii* stress (Bernatowicz and Pijanowska 2011) and for carbaryl + *Microcystis* stress (Cerbin et al., 2010). All of this reinforces the thought that it is at present still very difficult to generalize or extrapolate findings of interactive effects across species, natural stressors or chemicals. A better understanding of why some stressor combinations yield interactive effects and why others don't is a real research need, as this knowledge could eventually serve as a basis for developing predictive methods.

323 A first way forward could be to use knowledge of the stressor's modes of action, as it is, for example,
324 well-documented (at least for pesticides) that binary chemical mixture interactions often occur when
325 both chemicals target similar biological pathways or processes (Van Gestel et al., 2010). However, our
326 data do not seem to support this approach for predicting chemical x natural stressor interactions, as
327 they contradict with our expectation of interactive effects based on two well-known modes of action
328 that are common between Cd and *Microcystis* stress (oxidative stress and digestive activity, see also
329 *Introduction*). Our data rather corroborate findings of Barata et al. (2006), who found that classifying a
330 set of metals (Cu, Cd) and pyrethroid insecticides (λ -cyhalotrin, deltamethrin) according to some of their
331 known (primary and secondary) modes of action was not accurate in predicting interactive effects of
332 their binary mixtures on *Daphnia* responses at a higher level of biological organization (i.e., survival
333 and feeding responses). It seems that, if we ever want to predict which combinations of chemical and
334 natural stressors are likely to act interactively at the individual level (e.g. survival, reproduction) from
335 knowledge of the individual stressors' modes of action, we will likely need a more profound and more
336 complete understanding of how (pharmacological) modes of action translate to higher-level effects,
337 both in individual and mixed stressor exposures (Borgert et al., 2004).

338 A second, equally important way-forward could be to obtain a better understanding of inter-clonal
339 variation of interactive effects. Indeed, many published mixture studies are based on experiments with
340 single clones (see Holmstrup et al., 2010) and this could also be partly responsible for the variability of
341 interactive effects that is encountered in literature and for our current inability to generalize or
342 extrapolate findings across species and stressors. Two observations that we made when analyzing
343 our dataset on a clone-by-clone scale support this thought. First, quantitative analysis revealed a
344 relatively wide variation of log-deviations (both in direction and magnitude) between observed and IA-
345 predicted reproduction under combined Cd + *Microcystis* exposure among the clones (Figure 1).
346 Second, for one *D. magna* clone (i.e., OM2-24) out of a total of twenty tested clones this deviation was
347 also statistically significant, i.e. in a strictly statistical sense this clone experienced (as determined from
348 a two-way ANOVA) an antagonistic interactive effect from combined Cd + *Microcystis* exposure
349 (Figure 1, Table S1). Thus, if we suppose the hypothetical case that we had chosen to only investigate
350 this clone and that we would strictly rely on the result of the statistical test, we would have drawn
351 conclusions that would have been totally different to and anomalous with our general conclusion
352 (based on all 20 clones) that there is *on average* no interactive effect of Cd and *Microcystis* across

clones. Those two observations clearly caution against the use of a single clone for drawing generalized conclusions about chemical × natural stressor interactions and thus also for extrapolating results obtained with a single clone to make predictions for genetically diverse natural populations. Unfortunately, as mentioned before, the use of a single clone represents a situation that is still commonly found in published research of chemical + natural stressor mixtures. Example of such studies are: the above-cited carbaryl + *Microcystis* stress study in *D. pulicaria* (Cerbin et al., 2010), and several studies with *Daphnia* and other parthenogenetic organisms, such as the springtail *Folsomia candida* (summarized in Holmstrup et al., 2010). It should be clear that results from such single-clone studies should only be interpreted and used with a lot of caution in a chemical risk assessment context.

5. Conclusion

We investigated the effects on *D. magna* reproduction of combined exposure to Cd and *Microcystis*, the latter being a stressor predicted to become more prominent due to climate change. We found that the combined effects on *D. magna*, represented by a selection of 20 genetically different clones, were on average non-interactive. This was not expected based on some known shared modes of actions of Cd and *Microcystis*. This finding cautions against making predictions of interactive chemical + natural stressor effects from incomplete pharmacological mode of action / mechanism of toxicity knowledge. We did not find significant inter-clonal variation in the interactive effect of Cd and *Microcystis* when data for all clones were analyzed together in a 3-way ANOVA. However, the wide variation of deviations between observed and IA-model-predicted reproduction under combined Cd and *Microcystis* exposure, as well as the observation of a statistically significant antagonistic effect in one of twenty clones (when each clone was analyzed separately in a 2-way ANOVA), caution against the use of mixed stressor data obtained with single clones in risk assessment of chemicals and natural stressors, e.g. when extrapolating findings to the population level.

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Table 1. Overview of the origin of the *Daphnia magna* clones used.

Pond	Abbreviation	Geographical location	# clones used	Reference
Pond 15 of Knokke	KNO15	51°20'05.62"N, 03°20'53.63"E	3	Coors et al., 2009
Pond 17 of Knokke	KNO17	51°21'01.97"N, 03°19'49.58"E	2	Orsini et al., 2011
Langerode	LRV	50°49'42.08"N, 04°38'20.60"E	2	Rousseaux, 2011
Moorsel	MO	50°51'54.54"N, 04°32'05.87"E	2	Coors et al., 2009
Pond 2 of Oude Meren, Heverlee	OM2	50°51'47.82"N, 04°43'05.16"E	2	Coors et al., 2009
Pond 3 of Oude Meren, Heverlee	OM3	50°51'47.32"N, 04°43'05.16"E	3	Orsini et al., 2011
Pond 1 of Tersaert, Neerijse	TER1	50°49'22.98"N, 04°35'38.17"E	2	Orsini et al., 2011
Pond 2 of Tersaert, Neerijse	TER2	50°49'18.24"N, 04°36'04.50"E	2	Rousseaux, 2011
Pond 4 of Oud-Heverlee	ZW4	50°49'24.68"N, 04°39'53.46"E	2	Orsini et al., 2011

Table 2. Overview of measured dissolved cadmium (Cd) and dissolved organic carbon (DOC) concentrations (mean \pm SD) in fresh and 48h old medium (N=6).

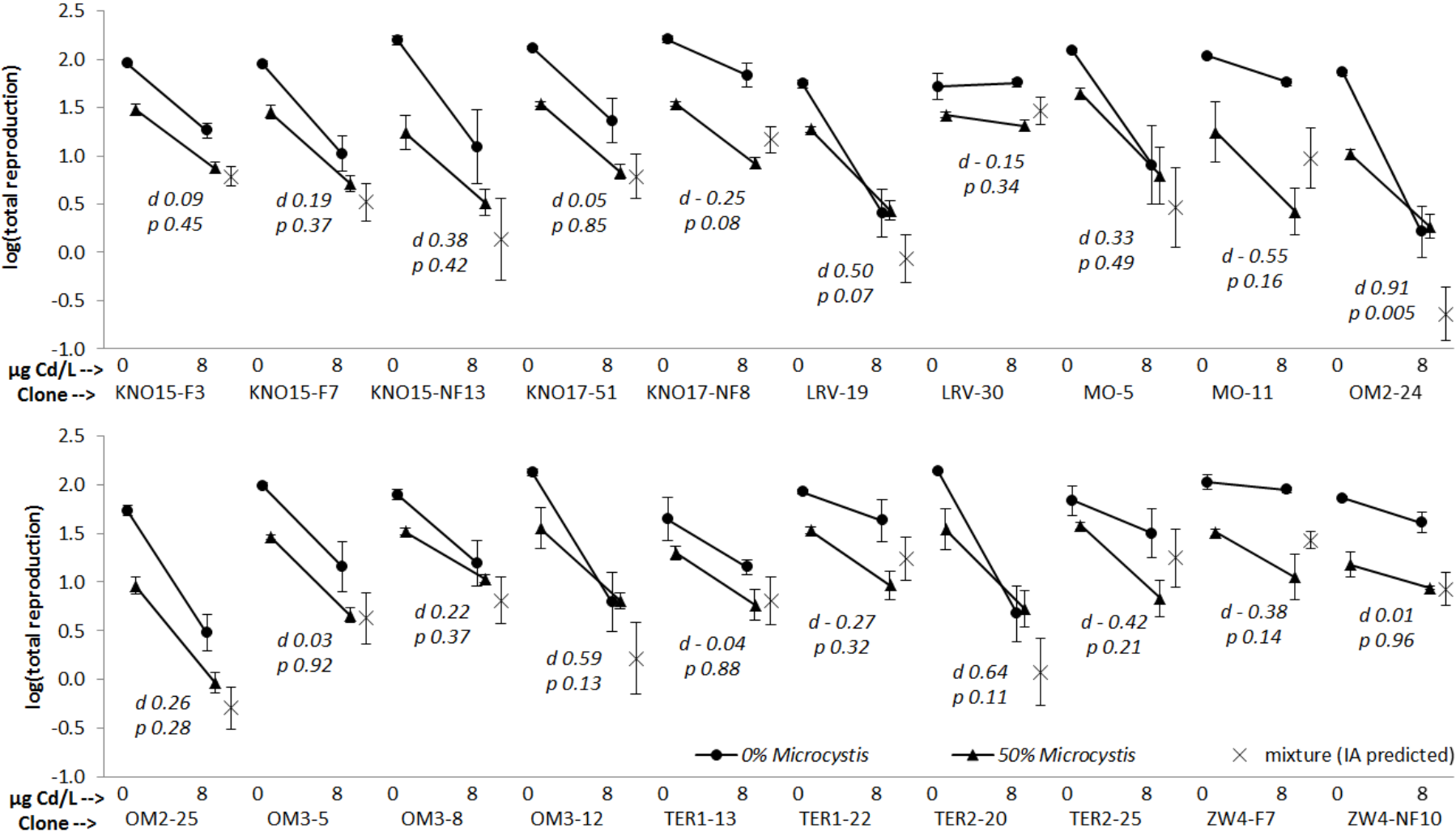
Treatment		Fresh medium	Old medium
Control	Cd ($\mu\text{g}\cdot\text{L}^{-1}$)	<DL	<DL
	DOC ($\text{mg}\cdot\text{L}^{-1}$)	4.5 \pm 0.3	5.3 \pm 0.5
Cd	Cd ($\mu\text{g}\cdot\text{L}^{-1}$)	8.9 \pm 0.9	7.2 \pm 1.2
	DOC ($\text{mg}\cdot\text{L}^{-1}$)	4.6 \pm 0.3	5.3 \pm 0.4
<i>Microcystis</i>	Cd ($\mu\text{g}\cdot\text{L}^{-1}$)	<DL	<DL
	DOC ($\text{mg}\cdot\text{L}^{-1}$)	4.5 \pm 0.3	5.5 \pm 0.3
Cd + <i>Microcystis</i>	Cd ($\mu\text{g}\cdot\text{L}^{-1}$)	8.9 \pm 0.9	6.9 \pm 1.3
	DOC ($\text{mg}\cdot\text{L}^{-1}$)	4.6 \pm 0.3	5.6 \pm 0.4

<DL: below detection limit of 0.07 $\mu\text{g}\cdot\text{L}^{-1}$.

539 Table 3. Summary of three-way ANOVA. Significant p-values at the 95% significance level are shown in bold.

	df	MS	F	P
Clone	19	1.815	5884.48	< 0.001
Cd	1	88.103	8.70	< 0.001
MC	1	44.876	44.34	< 0.001
Clone x Cd	19	1.046	215.12	< 0.001
Clone x MC	19	0.377	5.01	0.019
Cd x MC	1	0.585	1.81	0.105
Clone x Cd x MC	19	0.316	2.80	0.073
Error	647	0.209		

540 Cd: cadmium; MC: *Microcystis*; df: degrees of freedom; MS: mean square; F: F-value; p: p-value



543 Figure 1: Cd reaction norms based on total reproduction of the 20 distinct *Daphnia magna* clones under 0% (circles) and 50% (triangles) *Microcystis*
544 *aeruginosa* in the diet. Error bars represent standard error (some data points have a standard error that are smaller than the symbol size). The value next to *d*
545 is the \log_{10} deviation between IA-model predicted (Eq. 1) and observed reproduction in the Cd + *Microcystis* treatment for each clone. The value next to *p* is
546 the *p-value* for the Cd x *Microcystis* interaction term in the two-way ANOVA for each clone (see Table S1 for details).

547 **Supplementary Data**

548 www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S0166445X1300180X/1-s2.0-

549 [S0166445X1300180X-](http://www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S0166445X1300180X-)

550 [mmc1.docx/271226/FULL/S0166445X1300180X/372b641e2fdec83bb09a9c656a12f342/mmc1.docx](http://www.sciencedirect.com/science/MiamiMultiMediaURL/1-s2.0-S0166445X1300180X/372b641e2fdec83bb09a9c656a12f342/mmc1.docx)

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